# Production of pyranoanthocyanin pigments from cyanidin-derivatives with different glycosylation patterns for food colorant use

Authors: Megan Hoehn[*1],
Advisors: Jacob Farr[*], M. Monica Giusti[*]

[1]Hoehn.58@osu.edu
[*]The Ohio State University, Department of Food Science and Technology, 2015 Fyffe Road, Columbus, Ohio 43210

**Abstract**

Traditionally synthetic colorants have been used in food to ensure vibrant and stable colors, but as consumer acceptance decreases and health concerns over synthetic colorants arise, natural colorant use has increased. Anthocyanins (ACN) are natural colorants with limited stability. Pyranoanthocyanins (PACN) are recently discovered, anthocyanin-derived compounds that have increased stability and could function as safe natural food colorants. However, the conversion of the ACN to PACN is not well understood, limiting commercial food application. The purpose of this study was to understand how ACN chemical structure affects PACN formation. It was hypothesized that ACNs possessing more than two sugar moieties would have no PACN formation due to steric hindrance. Cyanidin-3-glucoside, Cyanidin-3,5-diglucoside, Cyanidin-3-galactoside, Cyanidin-3-glucoside-galactoside and Cyanidin-3-xyloside-glucoside-galactoside were isolated from plant sources and purified to test how location, type and number of sugar moieties affected PACN formation. Samples were combined with pyruvic acid and stored at 35℃ in pH 2.5 buffer. A HPLC-PDA was used to generate a chromatogram on day 0, 10 and 20. Spectral and color data were obtained on day 0 and 20. On day 20, all Cyanidin-3-glycosides showed PACN formation. The proportion of PACNs to total pigment ranged from 6.4% (Cyanidin-3-glucoside-galactoside) to 40.3% (Cyanidin-3-glucoside). Cyanidin-3,5-diglucoside experienced extensive pigment degradation by day 10 and had no PACN formation indicating pyruvic acid access to C5 on the ACN molecule is essential for PACN formation. A hypsochromic shift in the lambda maximum occurred in all samples ranging from 1.6-11.3 nm. A larger shift indicated more PACN formation. Preliminary data indicates C3 monoglycoside ACNs formed PACNs most efficiently, and the larger the substitution the less PACNs formed. ACN structural characteristics are an important parameter when optimizing PACN formation. Efficient production of PACNs could allow for a more stable naturally derived pigment for food industry use.

**Introduction**

Pyranoanthocyanins are anthocyanin-derived pigments (Figure 1) with an additional ring between carbon 4 and 5, and have been discovered within the last two decades. The pyranic ring forms naturally over time in aged wines and juices and contributes to their progressive shift from

red–purple toward red-orange (Freitas & Mateus 2011). The recent discovery of pyranoanthocyanins and the pigmentation that pyranoanthocyanins provide make them a beneficial area of research. Pyranoanthocyanins recent discovery and increased stability compared to anthocyanins make pyranoanthocyanins a possible solution to maintaining color quality in foods.

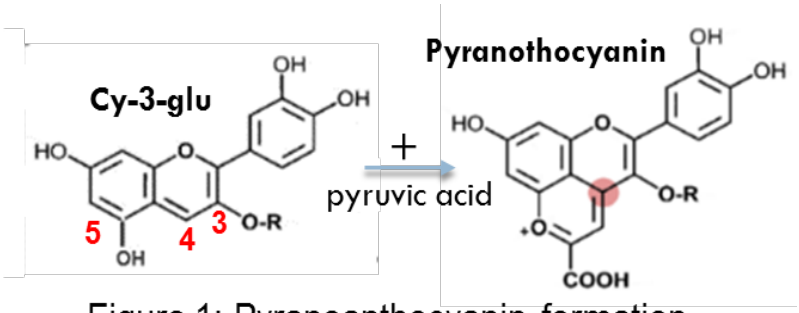

Figure 1: Pyranoanthocyanin formation

Studies have shown that pyranoanthocyanins retain color over a larger pH range compared to anthocyanins. Anthocyanins have been reported to lose 80% of their color intensity with a pH increasing from 1 to 5, while pyranoanthocyanins have a higher stability due to the protective pyranic ring, and likely different secondary equilibriums (Mazza and Miniati 1993). The new ring blocks the nucleophile attack of water, which prevents the formation of the colorless carbinol base (Rentzsch and others 2007). Another study has shown the formation of pyranoanthocyanins resulted in a hypsochromic shift in $\lambda_{max}$ from anthocyanins at ~510 nm to pyranoanthocyanins ~490 nm (He and others 2010). The increased stability of pyranoanthocyanins compared to anthocyanins was demonstrated in a study that compared the half-life of pyranoanthocyanins to anthocyanins. It was found that pyranoanthocyanins half-life increased by 8-13x compared to cyanidin-3-galactoside in the presence of ascorbic acid (Farr and Giusti 2016). The increased stability of pyranoanthocyanins and the change in the lambda maximum could make pyranoanthocyanins an important source of pigmentation in food.

For pyranoanthocyanin-related work, studies have mainly focused on the profiling of pyranoanthocyanins and their stability properties. Since pyranoanthocyanins are able to contribute to the pigmentation in aged wines then they potentially could act as a longer-term colorant for other food items. In order to use pyranoanthocyanins as a food colorant, the conversion from anthocyanin to pyranoanthocyanin must be understood. The objective of this study is to understand how substitution patterns on the anthocyanin aglycone influence the formation of pyranoanthocyanins.

The structural features of the anthocyanin may affect the conversion of the anthocyanin to the pyranoanthocyanin. For example, anthocyanins generally have a glycosylation at carbon 3.

This is also found, to a lesser extent, at carbon 5 (Bueno and others 2012). Anthocyanins can also have multiple sugars moieties at either site and can have acyl attachments extending from the glycosylation. The differences in the size and type of sugar moieties, as well as the number are all thought to possibly influence accessibility to carbon 4, which is critical for the formation of pyranoanthocyanins. There are currently no studies that investigate how the number, position, and type of sugar moiety influences anthocyanin to pyranoanthocyanin formation.

This study investigated the impact of several parameters on pyranoanthocyanin formation: (1) anthocyanin carbon 3 vs carbons 3 and 5 substitution with glucose, (2) the type of monosaccharide (carbon 3 glucoside vs galactoside substitution), and (3) the degree of substitution (carbon 3 mono vs di vs tri-saccharide). The objective of the study was to determine how the location, type, and size of anthocyanin sugar substitutions influenced pyranoanthocyanin formation.

## Hypothesis

It was hypothesized that as the number of sugar substitutions increased the amount of pyranoanthocyanin formation would decrease due to steric hindrance. It was believed that pyranoanthocyanins would form in the largest concentrations with one sugar substitution, in lesser amounts with two substitutions and not at all with three substitutions.

## Methods

Cyanidin-3-glucoside, Cyanidin-3,5-diglucoside, Cyanidin-3-galactoside, Cyanidin-3-glucoside-galactoside and Cyanidin-3-xyloside-glucoside-galactoside were generously provided by Greg Sigurdson. In brief, Cyanidin-3-glucoside and Cyanidin-3,5-diglucoside were obtained from partial hydrolysis of red cabbage anthocyanins using 1 N HCL and 15 minutes of boiling. The compounds were then purified using semi-preparatory HPLC (Durst and Wrolstad 2001). Cyanidin-3-galactoside, Cyanidin-3-glucoside-galactoside and Cyandin-3-xyloside-glucoside-galactoside were extracted from black carrot. The samples under went semi-preparatory HPLC to separate and purify the compounds (Durst and Wrolstad 2001).

A pH 2.5 buffer was made using citric acid and sodium phosphate. The pH was adjusted using HCl and NaOH. The pH was tested on day 0, day 10 and day 20 of the experiment to ensure the buffer remained stable. The concentrated pigments were dissolved in the pH 2.5

buffer to achieve a monomeric anthocyanin concentration of 500 mg Cyanidin-3-glucoside equivalents/L (Giusti and Wrolstad 2001).

Each compound was divided into 3 control vials and 3 pyruvic acid vials. The control vials contained 35.5 microliters of water and 1.0 mL of pigment. The pyruvic acid vials contained 35.5 microliters of pyruvic acid and 1.0 mL of pigment creating a 1:50 molar ratio of anthocyanin:pyruvic acid. The samples were stored in 2 mL HPLC vials in a 35°C incubator. The incubator ensured the samples had no exposure to light during incubation.

The samples were run on an HPLC-PDA (Shimadzu, Maryland, U.S.) on day 0, 10 and 20. An automated sampler injected 10 microliters of pigment into the HPLC column C18. The two solvents used were A: 4.5% formic acid (Sigma Aldrich St. Louis, Missouri) in HPLC grade water (Fisher Scientific, Hampton, New Hampshire) and B: HPLC grade acetonitrile (Fisher Scientific, Hampton, New Hampshire). A 1 mL/min flow rate was used. The gradient started with an isocratic flow of 6% solvent B for 17 minutes, raised to 15% solvent B at 45 minutes, up to 40% solvent B at 50 minutes, decreasing to 6% B by 55 minutes and remaining isocratic at 6% B until 60 minutes (Farr and Giusti 2016).

On day 0 and day 20 spectral data was collected using a plate reader (Microplate Reader, Molecular Devices, Sunnyvale, California). A 96 well plate was used and 65 microliters of pigment was injected into the wells. The pH 2.5 buffer was used in the blank wells. The data was analyzed to determine the lambda at maximum for the samples.

## Results and Discussion

The formation of pyranoanthocyanins from Cyanidin-3-glucoside over time was monitored by HPLC (Figure 2). The first peak represented the starting anthocyanin molecule and the second peak represented the pyranoanthocyanin. The chromatogram was analyzed to determine the percent area under the curve (in a max plot from 490 to 510nm) represented by pyranoanthocyanins as compared to the total pigment at a given time point (Figure 3). As time increased more pyranoanthocyanins were formed. On day 20 a small broad peak between 25 and 30 minutes appeared on the chromatogram, and plausibly represented formation of polymers.

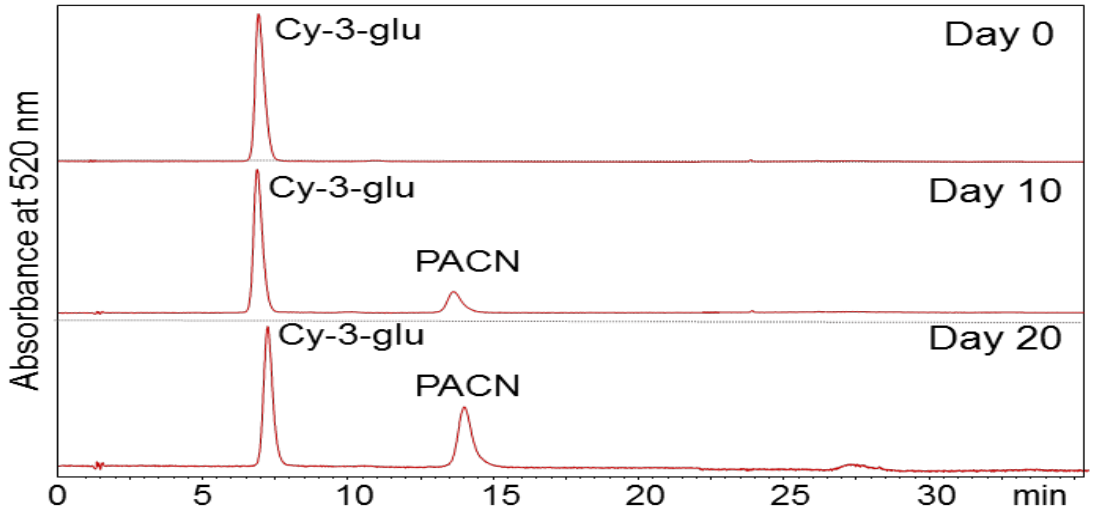


Figure 2: Pyranoanthocyanin formation from Cyanidin-3-glucoside and pyruvate in pH 2.5 buffer over 20 days at 35°C in the dark.

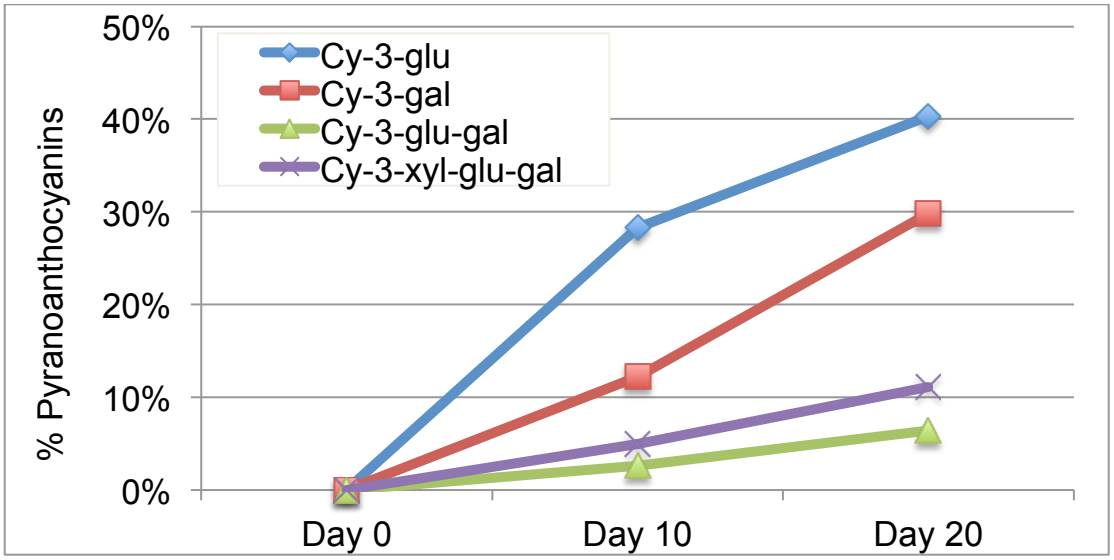


Figure 3: Percent pyranoanthocyanin to total pigment of various cyanidin (Cy) glycosylation patterns after incubation with pyruvic acid for 0, 10 and 20 days at 35°C

Cyanidin-3,5-diglucoside is not shown in Figure 3, because it had extensive pigment degradation by day 10 and no pyranoanthocyanin formation was observed. This was likely attributed to the sugar attached to carbon 5. Pyruvic acid attaches to the anthocyanin molecule at carbon 5, so a sugar could have blocked access to carbon 5 (Marquez, Serratosa & Merida 2012). The degradation and lack of pyranoanthocyanin formation with Cyanidin-3,5-diglucoside suggest that pyruvic acid access to carbon 5 on the anthocyanin molecule may be essential for pyranoanthocyanin formation.

A hypsochromic shift in the lambda maximum is characteristics of pyranoanthocyanin formation (Marquez, Serratosa & Merida 2012). Pyranoanthocyanins generally have a lambda maximum of 490-500 nm and represent a hypsochromic shift in the lambda maximum with respect to the starting anthocyanin (Marquez, Serratosa & Merida 2012). The results from this experiment aligned with expected results. A hypsochromic shift in the lambda maximum ranging from 1.6 - 11.3 nm occurred in all samples (Figure 4) consistent with pyranoanthocyanin formation from each of the four compounds evaluated, Cyanidin-3-glucoside, Cyanidin-3-galactoside, Cyanidin-3-glucoside-galactoside and Cyanidin-3-xyloside-glucoside-galactoside. A larger shift in the lambda maximum correlated with higher pyranoanthocyanin formation. Cyanidin-3-glucoside had the largest shift in the lambda maximum (11.3 nm), followed by Cyanidin-3-galactoside (3.7 nm). These results aligned with the results obtained from the chromatogram implying that simple anthocyanins formed pyranoanthocyanins most efficiently.

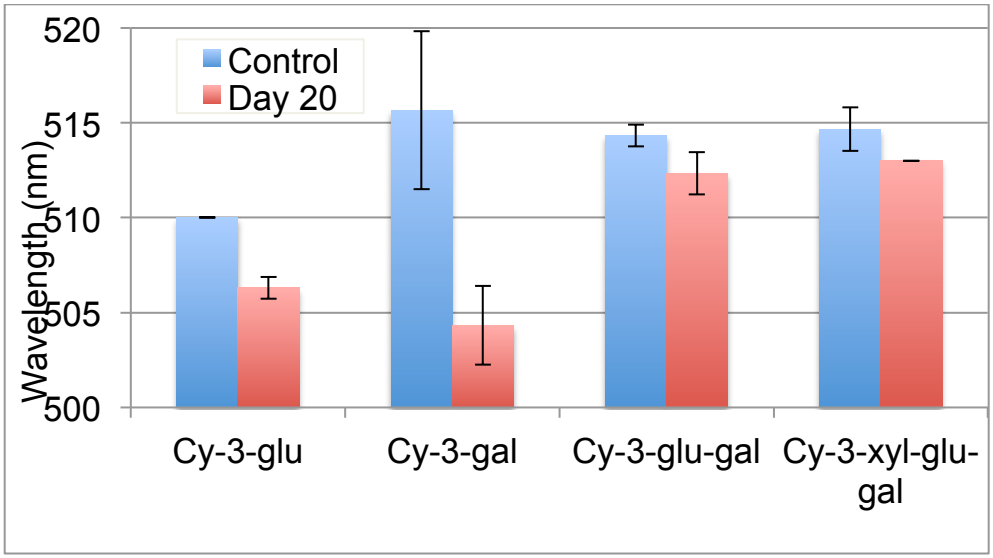


Figure 4: Changes in lambda maximum of Cyanidin-derivatives solutions incubated with and without pyruvic acid after 20 days at 35°C in the dark.

The results from Figure 3 were generated with an HPLC and the results from Figure 4 were generated with a plate reader. All results indicated that Cyanidin-3-glucoside and Cyanidin-3-galactoside formed the largest amounts of pyranoanthocyanins, implying simple anthocyanins formed pyranoanthocyanins most efficiently. However, Cyanidin-3-xyloside-glucoside-galatoside had a higher percent pyranoanthocyanin formation than Cyanidin-3-glucside-galacoside. This raised a new question on molecular orientation and pyranoanthocyanin

formation. The orientation of the sugar molecules may have an effect on formation. The different sugar orientations potentially create different steric affects, which would effect pyranoanthocyanin formation. As shown in Figure 5 sugars attach to the anthocyanin molecule differently. Some sugars are able to freely rotate and others have limited mobility. The mobility of the molecule changes the steric hindrance affect, which would affect pyranoanthocyanin formation. Further research should be done to look at the effect of molecular orientation on pyranoanthocyanin formation.

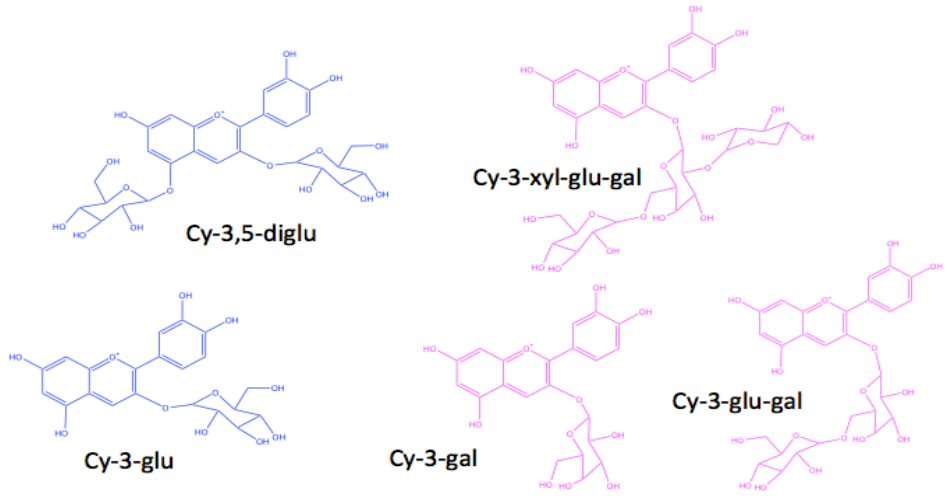


Figure 5: The attachment of the sugar molecules to Cyanidin. The starting anthocyanin molecules

## Conclusion

Cyanidin-3-glucoside had the highest pyranoanthocyanin formation after incubation for 20 days at 35°C in pH 2.5 buffer, at 40.3%, followed by Cyanidin-3-galactoside at 29.8% and they were also the pigments that showed the largest hypsochromic shift in the lambda maximum. Our results showed that cyanidin-3-monoglycoside formed pyranoanthocyanins most efficiently, and the larger the sugar substitution the less pyranoanthocyanins formed. The role of steric hindrance is likely critical in the kinetics of pyranoanthocyanin formation and could be used to efficiently produce the more stable pyranoanthocyanin. Efficient production of pyranoanthocyanins could allow for a more stable naturally derived pigment for food industry use.

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